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<p>(54) Title: METHOD OF AND APPARATUS FOR CELL PORATION AND CELL FUSION USING RADIOFREQUENCY ELECTRICAL PULSES</p>		
<p>(57) Abstract</p> <p>Disclosed are an apparatus and a method for the poration and fusion of cells using high-power radiofrequency electrical pulses. The electrodes of the apparatus can be hand held or part of integrated equipment with special containers for cells. The electrodes, which are positioned equidistant from each other, are attached to a high power function generator. The power function generator can apply a continuous AC electrical field and/or a high-power pulsed radiofrequency electrical field across the electrodes. The alternating electrical field induces cell congregation by the process of dielectrophoresis. The high-power pulsed radiofrequency electrical field porates or fuses the cells. The method has the ability to porate or fuse biological cells with a very high efficiency. The method can be used to fuse or porate a variety of cells including animal cells, human cells, plant cells, protoplasts, erythrocyte ghosts, liposomes, vesicles, bacteria and yeasts. The method has the unique ability to porate or fuse cells in very small or very large numbers. During the poration or fusions, a variety of chemical agents including DNA, RNA, antibodies, proteins, drugs, molecular probes, hormones, growth factors, enzymes, organic chemicals and inorganic chemicals can be introduced into these cells. The method can also be used to produce new biological species, to make hybridoma cells which produce animal or human monoclonal antibodies and to insert therapeutic genes into human cells which can be transplanted back into the human body to cure genetic diseases.</p>		

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METHOD OF AND APPARATUS FOR CELL PORATION AND CELL
FUSION USING RADIOFREQUENCY ELECTRICAL PULSES

Field of the Invention

This invention relates to the field of poration and fusion of biological cells by application of a high-power pulsed radiofrequency electric field. More particularly, it relates to permeabilizing and fusing cells in a wide variety of fields including gene transfection, micro-injection of cells, production of monoclonal antibodies and making new biological species by hybridization.

Background

Cell poration and cell fusion play a very important role in modern biotechnology. For example, one key procedure in genetic engineering is the introduction of exogeneous genetic material into a host cell. This insertion of genes is accomplished by either permeabilizing the cell membrane to allow entry of genetic material (i.e., gene transfection) or fusing the host cell with a cell containing the desired genetic material. Cell fusion is also important in the production of monoclonal

1 antibodies. The process of producing monoclonal
antibodies requires the fusion of antibody producing cells
with continuously dividing cancer cells. (Galfre, G. et
5 al., Nature 266:550-552 (1977); Lo, M. M. S. et al.,
Nature 310:794-796 (1984)). Additionally, one highly
effective method of delivering drugs which normally cannot
enter a cell is to fuse the cell with liposomes or red
blood cell ghosts that have been pre-loaded with specific
10 drugs. (Schlegel & Lieber, Cell Fusion, ed. by A. E.
Sowers, Plenum Press (1987)).

The conventional techniques of cell fusion rely
mainly on the actions of viruses (White, J. et al., J.
Cell Biol. 89:674-679 (1981)); or chemical agents such as
15 polyethylene glycol (Davidson, R. L. et al., Somatic Cell
Genetics 2:271-280 (1976)). Virus-induced and
chemical-induced fusion methods have many shortcomings.
Not only is the fusion yield often very poor, typically
less than 0.01%, but the standard fusion techniques may
20 also cause severe side effects on the fused cells, thus
greatly limiting their usefulness for many systems.

Alternative methods which induce cell fusion and
cell poration by electric fields have been developed.
(Pohl, U. S. Patent No. 4,476,004; Sowers, U. S. Patent
25 No. 4,622,302; Schoner, U. S. Patent No. 4,578,167;
Neumann, E. et al. Naturwissenschaften 67:414-415 (1980);
Zimmerman, U. and Nienken, J., J. Membrane Biol.
67:165-182 (1982); Bates G. W., et al., Cell Fusion,
Plenum Press pp. 367-395 (1987)). The basic principle of
30 these methods of electrofusion is to apply a pulsed high
strength direct-current (DC) electric field across the
cell. This DC field is usually generated by briefly
switching on a DC power source or by discharging a
capacitor. The applied DC field has a strength of several
35 kilovolts per centimeter. This external electric field
induces a large cell membrane potential. When the

1 membrane potential is of sufficient magnitude, a
reversible breakdown of a small area of the cell membrane
occurs. The breakdown results in the formation of
5 physical pores at the surface of the cell. This process
is called electroporation. Intracellular and
extracellular material can exchange through the pore while
it is open. After the DC field is removed, the pore will
normally reseal quickly. When a pore is created between
10 two closely adjacent cells a cytoplasmic bridge is formed
via the pore. When the DC field is turned off the pore
cannot reseal. Instead, the cytoplasmic bridge usually
begins to enlarge, eventually causing the two cells to
fuse.

15 Although the DC electrofusion method has been
used successfully for a number of biological cells,
including plant protoplasts (Zimmerman, U. et al.,
Biochem. Biophys. ACTA 641:160-165.(1981); Bates, G. W. et
al., Cell Fusion, Plenum Press pp. 479-496 (1987)); blood
20 erythrocytes (Sowers, A. E., J. Cell. Biol. 102:1358-1362
(1986); Chang and Hunt, Proceedings of the International
Symposium on Molecular Mechanisms of Membrane Fusion,
Buffalo, New York pp. 26 (1987); Stenger, D. A. and Hui,
S. W., J. Membrane Biol. 93:43-53 (1986)); tumor cells
25 (Lo, M. M. S. et al., Nature 310:794-796 (1984); Tessie,
J. et al., Science 216:537-538 (1982)); yeast cells
(Halfmann, H. J., et al., Archiv. Microbiol. 134:1-4
(1983)); and blastomeres and eggs (Kubiak, J. Z. and
Jarkowski, A. K., Exp. Cell Res. 157:561-566 (1985)),
30 there are still many limitations to the use of this
method. First, not all cell types can be fused with the
same ease. In fact many cell types are extremely
difficult to fuse with DC pulses. Second, there are many
unknown factors which influence fusion yield. Fusion of
certain cell types may be successful in one laboratory but
35 not in others. The DC pulse method is still more of an

1 art than a well understood procedure. Third, it is very
difficult to use the DC pulse method to fuse cells of
different sizes. This later problem occurs because the
5 membrane potential induced by the external DC field is
proportional to the diameter of the cell. Thus, the
induced potential is larger for bigger cells. It is
nearly impossible to chose a proper field strength of
external field in order to fuse cells of two different
10 sizes. When the external field is just sufficient to
cause membrane breakdown in the larger cell, it is
inadequate to induce a critical membrane potential in the
smaller cell. On the other hand, if the external field is
elevated to cause a membrane breakdown in the small cell,
15 the large potential induced in the larger cell will cause
an irreversible membrane breakdown and destroy the cell.

The present invention provides an improved method
of cell poration and cell fusion which overcomes the above
problems. Unlike the conventional electrofusion method
20 which employs DC pulses to induce membrane breakdown, the
present invention uses a pulse or pulses of radiofrequency
(RF) electric field to reversibly permeabilize cells and
induce cell fusion. The high-power RF field produces an
oscillating motion of the cell membrane through a process
25 of electro-compression. Permeabilization of the cell
membrane is caused by a combination of electrical
breakdown and a localized sonication induced from the RF
field. Thus, this oscillating electric field is more
effective in breaking down the cell membrane than a DC
30 field. Since this new method uses only physical means
(i.e., RF electrical energy) to induce cell poration and
cell fusion, it is free of biological or chemical
contamination. The present invention produces results in
seconds, provides much higher yields than conventional
35 methods, and has minimal biological side effects. Thus,
it is a clean, fast, efficient and safe method.

1 The improved efficiency of cell poration and cell
fusion offered by the method of this invention has a
particular significance in medical applications. One
5 example is to produce antibodies for therapeutic uses.
Since the human body usually rejects animal antibodies,
such therapeutic antibodies must be produced by hybridomas
of human cells; however, human hybridomas are extremely
difficult to form by conventional methods (including
10 electro-fusion by DC field). The method of the present
invention will help to improve the efficiency in forming
human hybridomas. Another example of medical application
of this method is gene therapy. Many genetic diseases can
be treated by inserting a therapeutic gene into the
15 patient's cells in vitro and then transplanting the cells
back to the patient's body. The conventional methods of
cell poration (including the DC field method) usually
require a large number of cells (typically 5-10 million
cells) to perform a gene transfection and, as a result,
20 are unsuitable for use in human therapy. In contrast, the
method of the present invention has been demonstrated to
be able to transfect cells in small numbers with high
efficiency, and will be highly useful for gene therapy.

Summary of the Invention

25 An object of the present invention is a method
for the poration of cells.

 An additional object of the present invention is
a method for the fusion of cells.

30 A further object of the present invention is a
device for the poration and fusion of cells.

 Another object of the present invention is a
method for inserting genetic materials into biological
cells.

35 A further object of the present invention is the
treatment of genetic disease by inserting therapeutic
genes into cells that are transplanted into diseased

1 patients.

Another object of the present invention is a method for the formation of hybridoma cells by the fusion
5 of cells with RF electric field.

An additional object of the present invention is a method which greatly enhances the efficiency of producing monoclonal antibodies.

Another object of the present invention is the
10 formation of a new species by the fusion of cells from different species using high-power RF pulses.

An additional object of the present invention is the introduction of chemicals and biological molecules into cells by the procedures of poration and/or fusion.

Thus, in accomplishing the foregoing objects
15 there is provided in accordance with one aspect of the present invention a method for poration of biological particles comprising the steps of placing a plurality of biological particles in solution between two electrodes
20 and applying a high-power pulsed RF oscillating field across the electrodes for porating the particles. The biological particles can either be suspended cells in solution or attached cells in cell culture. An additional embodiment of this method includes fusing the biological
25 particles by placing the suspended biological particles in a container which allows the biological particles to congregate before applying the pulsed RF field.

An alternative method includes fusing the biological particles by applying a low power (e.g., 100 to
30 400 V/cm) alternating current (AC) electrical field before and/or after the pulsed RF oscillating field. The low-power electric field can cause the particles to move dielectrophoretically to form "pearl chains".

The biological particles can be a variety of
35 materials including biological cells (human, animal or plant cells), liposomes, vesicles, erythrocyte ghosts,

1 protoplasts, bacteria, and yeasts.

The pulsed RF field applied for the poration and fusion of cells can be an oscillating field of a single frequency or a mixed frequency. The RF oscillating field may be in the frequency range of 10 KHz to 100 MHz with a pulse width of about 1 μ sec to 10 msec and a pulse amplitude of up to about 20 KV/cm. In a preferred embodiment the RF oscillating field is about 0.02 to 10 MHz and the pulse width is about 20 to 2000 μ sec and the pulse amplitude is about 2-10 KV/cm. The wave form of the RF field may be sinusoidal, triangular, sawtooth, or square waves.

Another aspect of the present invention is the fusion of cells for the formation of new species, the introducing of chemical agents and natural or man-made genetic material into cells, and the formation of hybridoma cells. By the appropriate selection of cell types and materials new species can be formed either by the combining of genetic material from two different species by the fusion of their cells, or by the isolation or synthesis of the genetic material, and then the introduction of the genetic material into cells by either poration or fusion. Hybridoma cells are made by the fusion of antibody producing cells with continuously dividing cancer cells. Chemicals, drugs, DNA, RNA and other molecules can be introduced into cells by preloading vesicles, liposomes or erythrocyte ghosts before fusion with target cells.

Another aspect of the present invention is a device for the poration or fusion of biological particles comprising a container of non-conducting material capable of holding liquid and including an access port for receiving the biological particles. The device also includes electrodes positioned equidistant from each other and inserted into the container. A high-power function

1 generator is attached to the electrodes and is capable of
generating a RF electric field and/or an alternating
electric field. In one embodiment the container is shaped
5 to allow the biological particles to congregate.

An additional aspect is a device for poration and
fusion of biological particles comprising a glass chamber
and used with an optical microscope for observation of the
poration and fusion of cells.

10 A further aspect is a cell poration and fusion
device which can be hand-held. This device includes a
handle and equidistant electrodes. The electrodes can be
side-attached or bottom-attached and can be designed in a
variety of shapes including rings, circles, double
15 helices, squares, ellipses, concentric rings, concentric
squares, interdigitating arrays, spirals and parallel
plates.

Other and further objects, features and
advantages will be apparent from the following description
20 of the presently preferred embodiment of the invention
given for the purpose of disclosure when taken in
conjunction with the accompanying drawings.

Brief Description of the Drawings

25 The invention will be more readily understood
from a reading of the following specification by reference
to accompanying drawings, forming a part thereof, where
examples of embodiments of the invention are shown and
wherein:

30 Figure 1 is a schematic of one form of the
present invention using a chamber which allows for the
congregation of cells by gravity. 1A is a top view of the
device and 1B is a cross-sectional view of the device
showing the fusion chamber.

35 Figure 2 is a graph of examples of the
radiofrequency (RF) pulses used in the present invention.

1
2A is a single-frequency symmetrical RF pulse, 2B is an
asymmetrical RF pulse, 2C is a multiple-frequency RF
pulse, 2D is consecutive RF pulses of different
5 frequencies and 2E is a low-power AC field followed by a
high-power RF pulse followed by a low-power AC field.

Figure 3 is a schematic of one form of the
present invention showing a large volume chamber for cell
poration and/or cell fusion. 3A is a top view of the
10 fusion chamber and 3B is a cross-sectional view showing
the arrangement of electrodes in the chamber.

Figure 4 is a schematic of a chamber for cell
poration and/or cell fusion for optical microscopic
observation. 4A is a elevational view of the chamber and
15 4B is a cross-sectional view of the chamber.

Figure 5 is a schematic of a hand-held device for
cell poration and/or cell fusion using a side contact
configuration. 5A shows an elevational view of the device ;
and 5B shows a cross-sectional view of the electrode
20 inserted inside the cell container.

Figure 6 is a schematic of a double helical
design for the side-contact electrode assembly. 6A shows
a elevational view of the helical design for the electrode
assembly and 6B shows a side view of the same assembly.

25 Figure 7 is a schematic view of a segmented ring
design for the side-contact electrode assembly. 7A shows
an elevational view of the electrode assembly, 7B shows
the connection of the electrode rings in the electrode
assembly and 7C is a top view of a single electrode ring.

30 Figure 8 is schematic of a rectangular electrode
assembly for cell poration and cell fusion. 8A shows an
elevational view of the electrode assembly and 8B shows
the connection of the electrode squares in the electrode
assembly.

35 Figure 9 is a schematic of a cell fusion and cell
poration device with a bottom-contact configuration of

1 electrodes.

Figure 10 is a schematic of the double spiral design for the bottom-contact electrode assembly. 10A shows a elevational view and 10B shows top view of the electrode.

Figure 11 is a schematic view of a concentric ring design for the bottom-contact electrode assembly. 11A shows an elevational view and 11B shows a top view of the electrode.

Figure 12 is a schematic view of different designs for a bottom-contact electrode assembly. 12A is a top view of a square spiral assembly, 12B is a top view of a concentric square assembly, 12C is a top view of an interdigitating array assembly and 12D is a top view of a parallel plate assembly.

Figure 13 is a schematic of a probe for cell poration and cell fusion of a small number of cells using the RF method. The exterior of the metal electrode is designed to fit inside the wells of a 96-well cell culture plate. 13A is a three-dimensional view of the probe, 13B is a cross-sectional view and 13C is a fragmentary elevational view of the electrode.

Figure 14 is a block diagram of the apparatus which provides the source of the AC field for dielectrophoresis and the high-power RF pulses for cell poration and/or cell fusion.

Figure 15 is an electron micrograph showing the surface of a human red blood cell following RF poration treatment. Three RF electric field pulses were applied with a one second interval. The cells were rapidly frozen in liquid freon which was cooled by liquid nitrogen (Temperature 90°K). The frozen sample is examined by freeze-fracture electron microscopy. Magnification 50,000X.

Figure 16 is fluorescent micrographs showing the

1 events of fusion between human red blood cells. Red cells
were lined up in pearl chains by the process of
dielectrophoresis. Roughly 10% of the cells were
5 prelabelled with a fluorescent dye which produced bright
images under a fluorescence microscope. The unlabelled
cells could not be seen. 16A shows how the cells looked
before applying the RF pulses. No transfer of dye between
labelled and unlabelled cells was seen. 16B shows how the
10 cells looked 4 minutes after 3 RF pulses (40 μ sec wide,
300 KHz, 5 KV/cm) were applied. Some of the labelled
cells fused with their unlabelled neighboring cells,
allowing the fluorescent dye to transfer between them.

Figure 17 is a graph showing the measured fusion
15 yield between human red blood cells using three electrical
pulses (4 KV/cm, 100 μ sec). The fusion yield is shown
to vary with the oscillating frequency.

Figure 18 is a time series of optical micrographs
showing the fusion of a xanthophore cell with a fish tumor
20 cell induced by pulsed RF fields. 15A is before fusion
but after the xanthophore (marked by the arrow) was
brought into close contact with two tumor cells by
dielectrophoresis. 15B is two minutes after application
of the RF pulses showing that the xanthophore has already
25 begun fusing with one of the tumor cells. 15C is 4
minutes after application of the RF pulses showing that
the xanthophore and tumor cell have completely merged into
a single round cell.

DETAILED DESCRIPTION

30 In the description which follows, like parts are
marked throughout the specifications and drawings with the
same referenced numerals. The drawings are not
necessarily to scale and certain features of the invention
may be exaggerated in scale or shown in schematic form in
the interest of clarity and conciseness. It will be
35 readily apparent to one skilled in the art that various

1 substitutions and modifications may be made to the
invention disclosed herein without departing from the
scope and spirit of the invention.

5 One embodiment comprises a method for poration of
biological particles comprising the steps of placing the
biological particles in solution between two electrodes
and applying a pulsed radiofrequency (RF) oscillating
10 electric field across the electrodes, Fig. 1. A variety
of biological particles can be used including biological
cells, erythrocyte ghosts, liposomes, protoplasts,
bacteria and yeasts. The biological particles can be
suspended cells in solution or can be attached cells in
cell culture.

15 When a cell is placed in an electric field, an
electrical potential is induced across the cell membrane.
For a spherical cell, the membrane potential induced by an
external electric field is

$$V_m = 1.5 rE \cos \theta \quad (1)$$

20 where r is the radius of the cell, E is the
strength of the external field and θ is the angle
between the direction of the external field and the normal
vector of the membrane at the specific site.

The induced electric field within the membrane is

25 $E_m = V_m/d = 1.5 (r/d) E \cos \theta \quad (2)$

where d is the thickness of the membrane. Since
 d is much smaller than r (d is about 6×10^{-7} cm while r
is in the order of several microns), E_m is about 1000
fold larger than the applied field, E . The large electric
30 field within the membrane produces two effects. First, it
exerts a strong force on the phosphate head group of the
lipid molecules in the membrane and tends to move them in
the direction of the field. Secondly, it compresses the
membrane. When the external electric field oscillates,
35 the lipid molecules within the membrane also undergo an
oscillating motion.

1 In this arrangement, the cell itself functions as
an antenna and the membrane is a transducer which converts
the electrical oscillation into a mechanical oscillation.
5 Thus, it is possible to generate an ultrasonic motion in
the cell membrane by applying an external RF field.
Because the induced potential at a given site of the
membrane is a function of the angle between the
orientation of the membrane and the electric field vector,
10 the induced potential is not uniform over the entire cell
surface. The applied energy is focused at the poles of
the cell, that is, at $\theta = 0^\circ$ or 180° . Because the
amplitude of the external field can be adjusted such that
there is sufficient sonication power to break down the
15 cell membrane at the poles but not at other parts of the
membrane, the sonication can be localized. Experiments
indicated that this localized membrane breakdown induced
by the externally applied pulsed RF field is reversible.
That is, the pore(s) induced by the RF field reseal
20 quickly (within minutes) after the field is turned off.
Furthermore, most of the cells apparently stay viable.

Such temporary permeabilization of the cell
membrane is called cell poration. During this time period
when pores are formed, a brief exchange of intracellular
25 and extracellular materials occur. Many molecules,
including drugs, antibodies, and gene segments, which
normally cannot penetrate the cell membrane, can enter the
cell through the temporarily opened pores that were
induced by the pulsed RF field.

30 Another embodiment of this invention comprises a
method for fusing cells. In order for biological
particles to be fused, they must be in close proximity.
When cells are in close proximity they are said to
congregate. Two alternative procedures may be used to
congregate the cells before fusion. In one, a container
35 with a shape that allows the biological particles to

1
congregate by gravity is used. For example, the bottom of
the container can be made in a concave shape (see Figs. 1
and 3). This allows the cells to congregate. When the
5 cell membranes are permeabilized by the applied RF field,
the closely adjacent cells can form cytoplasmic bridges.
This process results in the fusion of cells.

Alternatively, a low amplitude continuous
alternating current (AC) electrical field can be applied
10 across the two electrodes. The frequency ranges from
about 60 Hz to about 10 mega Hz. Typically a 100-400 V/cm
field strength is used. Under the low amplitude AC field
the cells act as dipoles and line up parallel to the
field, eventually forming a long chain of cells which
15 appear like "pearl chains". This process is called
"dielectrophoresis" (Schwan, H. P. and Sher, L. D., J.
Electrochem. Soc. 116:22C-26C (1969); Pohl, H. A. et al.,
J. Biol. Phys. 9:67-86 (1981)). Formation of this pearl
chain normally takes about a few seconds to one minute.

20 The present invention uses a pulsed RF field to
porate and/or fuse cells and has a clear advantage over
the conventional electro-fusion method that uses a pulsed
DC field. First, the RF field is a much more efficient
means of transmitting energy to the cell membrane than the
25 direct current field. The present invention utilizes a
localized sonication to break down the cell membrane.
This method is much more effective than the DC pulse
method which relies solely on the electrical breakdown.
The cell membrane is composed of macromolecules which have
30 characteristic frequencies of thermal motion. When the
frequency of the applied oscillating field matches one of
these natural frequencies, a condition of resonance is
reached, and the efficiency of energy transfer is greatly
enhanced. In real biological cells the resonance peak can
35 be very broad. The pulsed radiofrequency field can be
carefully varied to achieve the proper resonant frequency

1 for the cells of interest. Consequently, the ability to
induce membrane breakdown will require less power than
using a direct current field and results in less risk of
5 irreversibly damaging the cell.

Second, this invention overcomes the difficulties
encountered when the conventional methods are used to fuse
cells of different size. In order to produce an
electrical breakdown of the cell membrane, the
10 field-induced membrane potential must exceed a certain
critical value, V_c (typically 1 volt). Such breakdown
can be reversible, and the membrane will reseal after the
external field is turned off if the induced membrane
potential is not too much larger than V_c . The cell
15 normally remains viable after such reversible breakdown.
On the other hand, if the induced potential is much higher
than V_c , the membrane breakdown is irreversible, the
cell is permanently damaged, and will not remain viable.

From Eq. 1 it can be seen that when cells of
20 different sizes are placed inside an electric field, the
induced membrane potential is higher for the larger cell
than for the smaller cell. This size-dependence of
membrane potential causes a problem when attempting to
fuse cells of different sizes using a DC field. Assume
25 that two cells, A and B, are to be fused and that the
radius of cell A, r_a , is about twice as big as the
radius of cell B, r_b . In order to cause a reversible
membrane breakdown in cell B, the applied external field
must be sufficient so that $1.5 E r_b$ is greater than
30 V_c . However, the same applied electric field will
induce a much larger V_m in Cell A, and will cause an
irreversible breakdown of the membrane leading to damage
to this cell. Thus it is very difficult to use direct
current pulses to fuse cells of significantly different
35 sizes.

This problem can be solved by applying a pulsed

1 radiofrequency field. When the applied field is a
radiofrequency oscillating field instead of a DC field,
the amplitude of the induced membrane potential is a
5 function of the frequency. The membrane potential
predicted in Eq. (1) is derived under the steady state
condition. The induced potential does not arise
instantaneously upon the application of the external
field. If the external field is stationary, the membrane
10 potential will reach V_m given a sufficient time. The
time required to establish this steady state membrane
potential is called "relaxation time", or τ , which is
given by

$$1/\tau = 1/R_m C_m + 1/r C_m (R_i + 0.5R_e) \quad (3)$$

15 where R_m and C_m are specific resistance and specific
capacitance of the membrane, and R_i and R_e are the
specific resistances of the intracellular medium and the
extracellular medium, respectively. (C. Holzapfel et al.,
J. Membrane Biol., 67:13-26 (1982)). For a cell of
20 several microns in diameter, τ is typically in the order
of 1 μ sec.

Since R_m in most cells is very large, for
practical purposes, eq. (3) can be simplified to

$$\tau = r C_m (R_i + 0.5R_e) \quad (4)$$

25 Thus the relaxation time is approximately proportional to
the radius of the cell.

Because the build-up of the membrane potential
requires a time period characterized by the relaxation
time τ , the membrane potential induced by a RF field is
30 frequency dependent. If a radiofrequency field is applied
at a frequency smaller than $1/\tau$, the membrane potential
has no problem in following the external field. The
applied field will produce a 100% cellular response in
 V_m . On the other hand, if the frequency of the applied
radiofrequency field is greater than $1/\tau$, the membrane
35 potential cannot catch up with the changes in the applied

1 field, and the response of the membrane potential will be less than 100%. In general, the maximum membrane potential induced by a RF field is

$$5 \quad V(\omega) = 1.5 rE \cos \theta X(\omega) \quad (5)$$

where r , E and θ have the same meaning as in Eq. (1), ω is the angular frequency, and $X(\omega)$ is a function of the frequency such that

$$10 \quad X(\omega) = [1 + (\omega\tau)^2]^{-1/2} \quad (6)$$

when $\omega < 1/\tau$, $X(\omega)$ is near unity.

When $\omega > 1/\tau$, $X(\omega)$ decreases very rapidly with increasing frequency.

This frequency dependent effect can be used to fuse cells of different sizes. From Eq. (4), τ of the cell is roughly proportional to r . Thus the larger cell will have a longer τ . To fuse the A and B cells, a pulsed RF electric field that has a frequency ω is applied such that

$$20 \quad 1/\tau_a < \omega < 1/\tau_b \quad (7)$$

Since the frequency is less than $1/\tau_b$, $X(\omega)$ approaches unity for cell B and thus the field will produce a full effect on the small cell. On the other hand, since the frequency is greater than $1/\tau_a$, the induced membrane potential in cell A cannot fully follow the variation of the applied field, that is, $X(\omega)$ in cell A is less than unity. Thus, in a pulsed radiofrequency field, the effect of the stimulating field sensed by the small cell is greater than the effect on the large cell. Consequently, a pulsed radiofrequency field can be applied which induces a reversible breakdown of the membrane of the small cell without irreversibly damaging the larger cell.

One embodiment of a device 10 for poration and/or fusion of biological particles is shown in Figure 1. It is a fusion chamber which includes a non-conducting

1 container 13 for holding the solution 16 of biological
particles 19. The container has a slightly concave bottom
22 so that biological particles 19 will congregate, under
5 gravity, between the electrodes 25. The electrodes 25 are
a pair of equidistant metal wires or metal bands made of
nontoxic material, such as platinum or surgical stainless
steel. The electrodes can be parallel wires or can be in
almost any shape or design. The container 13 has an
10 access port 28 wherein biological particles 19 can be
added or removed.

To induce cell-poration or cell-fusion, a high
power function generator 31 generates one or many high
power RF pulses which are applied through the pair of
15 electrodes 25. The pulse shape can include one of those
shown in Fig. 2. In Fig. 2A, the pulse is a symmetrical
RF oscillation with a single frequency. In Fig. 2B, the
RF pulse consists of a single frequency asymmetrical
sinusoidal wave. In Fig. 2C, the RF pulse contains a
20 mixture of sinusoidal waves of multiple frequencies (in
this example, two frequencies). In Fig. 2D, alternating
sinusoidal pulses of different frequency are used. In the
preferred embodiment, the pulse shown in Fig. 2B is used,
because it allows the applied energy of the field to be
25 used more efficiently in inducing cell poration or
fusion. Although the preferred RF electric field wave
form is sinusoidal, other wave forms with repetitive
shapes can be used. For example, triangular waves, square
waves and sawtooth waves can be used to fuse or porate
30 cells of different types.

One skilled in the art will readily recognize
that the parameters of the pulsed field are changed to
accommodate the characteristics of the different
biological samples. The radiofrequency within the pulse
35 may vary over the complete radiofrequency range of 10 KHz
to 100 MHz. Typically a value in the order of 0.02 to 10

1 MHz is used for the poration and/or fusion of biological cells.

5 The width of the pulse may vary from about 1 μ sec to 10 msec. In the preferred embodiment approximately 20 μ sec to 2 msec is used.

10 The field strength is controlled by varying the pulse amplitude. For fusion and poration of cells the range of 1 to 20 kV/cm is employed. In the preferred embodiment pulses of field strength up to about 10 KV/cm are used.

15 The pulse can be a single pulse, a train of pulses or multiple trains of pulses. A train of pulses are multiple pulses with an interval in between; for example, a series of ten pulses 0.5 milliseconds in width each pulse separated by 0.5 seconds. In some instances such as the fusion of HL-60 cells, the maximum fusion yield is enhanced by applying multiple pulses.

20 The RF pulses used for cell-poration and cell-fusion are similar. The main difference is that in cell fusion, the cells need to congregate (be brought into close proximity) before the high power RF pulse is applied. Furthermore, the cells must be maintained in close proximity after application of the RF pulse. The above described device brought the cells together by
25 gravitational congregation. An alternative, and more efficient method of cell aggregation is dielectrophoresis, where a continuous alternating current (AC) electric field is applied across the electrodes before and/or after the application of the high-power RF pulse. The amplitude of
30 this continuous AC field is typically in the range of 100 to 400 V/cm. Its frequency may vary from about 60 Hz to about 10 MHz. During cell fusion in the preferred embodiment the actual electric field applied across the electrodes may look like that shown in Fig. 2E.

35 Another device for poration and/or fusion of

1 larger volumes of cells is shown in Fig. 3. An array of
equidistant electrodes 25 instead of a single pair of
electrodes is used to apply the AC field and the pulsed RF
5 field. The bottom of this fusion chamber can be either
flat or slightly concave. It is made of transparent
material such as glass or clear plastic. This chamber can
be placed on top of an inverted optical microscope so that
the events of cell fusion and/or cell poration can be
10 directly monitored. Since the effects of different
experimental conditions can be assayed in a timely manner
with the design, it will be useful for establishing the
optimal condition for cell fusion and/or cell poration.

The electrodes can be arranged in any pattern, as
15 long as they are maintained equidistant from each other.
In the preferred embodiments the patterns have included
interdigitating array, concentric circles and double
spirals.

Another preferred device 10 for cell poration and
20 cell fusion is shown in Fig. 4. This device 10 is
designed to allow observation of cell fusion under an
optical microscope using a small volume of cell
suspension. This device is formed by two glass plates 34
separated by spacers 37 of approximately 0.3 mm thickness,
25 with the cell suspension 19 sandwiched between the glass
plates 34. In one embodiment thin glass plates such as
cover slips are used. Electrodes 25 are two parallel
platinum wires which are about 0.5 mm apart. The platinum
wire electrodes 25 are connected to a high-power function
30 generator 31. The high-power function generator can
generate both alternating current electric fields and
pulsed radiofrequency fields. An inlet tubing 41 and an
outlet tubing 44 are used to insert and remove cells from
the space between the electrodes.

35 Another embodiment of the present invention for
cell poration and cell fusion is shown in Fig. 5. The

1 purpose of this device is to porate or fuse a very large
volume of suspended biological particles; including
biological cells, protoplasts, bacteria and yeasts. This
5 device 20 is designed for ease in application,
maintenance, and cleaning. The cell suspension is
contained in a non-conducting cylindrical container 13.
The electrode assembly 50 is attached to an insulating
handle 47. To porate or fuse the suspended cells, the
10 electrode assembly is lowered into the cell container 13
by manipulating the handle 47. The electrodes 25 are
connected to the high-power function generator 31 by a
connection means 49. The AC field for cell fusion and the
high power RF pulses for cell poration and/or cell fusion
15 are then applied through the electrodes 25 in the
electrode assembly 50.

In this device 20 the electrode assembly 50 is a
vertical cylinder 53 and metal electrodes 25 are exposed
at the side (i.e., the cylindrical surface). The cylinder
20 can be any non-conducting material, for example, glass,
plastic, or teflon. When the electrode assembly 50 is
lowered into the cell container 13, the suspended cells 19
are displaced and form a thin layer of cell suspension 19
surrounding the electrode assembly 50. Thus, all cells
25 are in close proximity of the electrodes. When an
electrical potential is applied across the electrodes, the
cells are exposed to the electric field.

One design of the electrode assembly 50 is shown
in Fig. 6. Two metal wires or bands are coiled to form a
30 double helix electrode 25. The helices are identical in
shape except one is positioned between the other. These
two helices are attached to a cylindrical support 53. The
spacing between these two helices 25 is kept constant.
Thus, when an electrical potential is applied across the
two metal wires, the amplitude of the electric field
35 generated between the two helices is uniform along their

1 entire length.

Another embodiment of the electrode assembly 50 for cell poration and cell fusion is shown in Fig. 7.

5 Here the electrode 50 assembly is comprised of a stack of metal ring electrodes 25 separated by non-conducting insulating spacers 53 of fixed thickness. These ring electrodes 25 are connected together in an alternating fashion to form two sets of electrodes 25, each of which
10 is then connected to the output terminals of the high-power function generator. The rings have an attachment means 56 and a hollow area 59 for the passage of the wire to the alternate electrode 25.

The electrodes 25 do not have to be circular, but
15 can be any shape. Shapes which can be used include circular, rectangular as in Fig. 8 or elliptical.

Another embodiment for cell poration and cell fusion is shown in Fig. 9. The cell suspension 19 is contained in a non-conductive container 13. An electrode
20 assembly 50 is attached to a handle 47 which can be used to manipulate the position of the electrodes. Unlike the previous devices, the electrodes of this embodiment are exposed at the bottom of the electrode assembly 50. This device is thus particularly useful in porating and/or
25 fusing cultured cells that attach to the bottom of culture dishes.

One design of the bottom-contact electrode assembly 50 is shown in Fig. 10. The electrode assembly 50 consists of two spirals of metal bands, which serve as
30 the "ground" (-) and "high voltage" (+) electrodes 25. The two spirals are positioned in such a way that the spacing between each spiral is maintained constant. The equal spacing arrangement ensures that an applied electric field across the two electrodes 25 is uniform in strength
35 throughout the entire area covered by the electrode assembly.

1
In addition to the spiral design, other
configurations including, multiple concentric rings,
rectangular shapes, interdigitating arrays, parallel
5 plates or elliptical shapes can be used (see Figs. 11 and
12). The rings or shapes connected in alternating fashion
into two groups. One group of these rings or shapes is
connected to the "ground" (-) terminal, while the other
group of rings or shapes are connected to the "high
10 voltage" (+) terminal of the high-power function
generator. The spacing between the rings or shapes is
constant so that the strength of the electric field
generated between the adjacent rings or shapes is uniform
throughout the entire assembly. In the bottom-contact
15 electrode assemblies, the electrodes can be wires, plates
or bands. In the preferred embodiment, the width of the
electrodes is greater than the depth of the cell
suspension.

Another embodiment of the present invention for
cell poration and cell fusion is shown in Figs. 13A-C.
20 The probe 20 allows cell fusion or gene transfection for a
small volume of cell suspension. The probe 20 will fit
into a flat-bottomed 96-well cell culture plate, for
example Corning model 25860. The probe 20 includes two
coaxial electrodes 25. The inner electrode 25a is a solid
25 cylinder and the outer electrode 25b is a hollow tube.
The coaxial electrodes 25 can be made of a variety of
conductive materials. In the preferred embodiment, the
coaxial electrodes 25 are made of stainless steel. The
coaxial electrodes 25 are attached to a nonconductive
30 insulating holder 54 preferably made of teflon or plastic.

The gap between the inner 25a and outer 25b
coaxial electrodes may vary from about 0.5 to 2.0 mm. In
a preferred embodiment, the electrode 25 has a 0.7 mm
gap. With this probe 20 the total volume of suspended
35 cells to be fused or porated is about 80 μ l and it is

1 possible to do cell fusion or cell poration with as little
as 20 μ l of cell suspension.

The probe 20 has a handle 47 made of
5 non-conductive material, preferably teflon. Holding means
55, hold the outer electrode 25a in place.

This design has several advantages. Besides
allowing the use of small volumes of cell suspension for
cell fusion or cell poration, it is also simple to use and
10 highly cost-effective. Unlike most commercial machines
which require one cuvette to transfect one cell sample,
this probe can serially transfect many cell samples using
plates with multiple wells.

Another embodiment of the present invention for
15 cell poration and cell fusion is shown in Figure 14. This
figure shows the block diagram of the high power function
generator which generates both the AC field for
dielectrophoresis and the high power RF pulses for cell
fusion and/or cell poration. The switching between the AC
20 field and the RF field is controlled by a mercury wetted
relay. The RF pulses are generated by gating the output
of a radiofrequency oscillator and then passing through a
MOSFET power amplifier, the power output of which may be
as high as twenty kilowatts.

25 Alternatively the AC field and the pulsed RF
field can be generated by synthesizing the required
electrical wave with a digital computer and amplifying
these wave forms using a power amplifier. In this
embodiment the protocol can be controlled entirely by the
computer and thus no switching relay is needed. This
30 computer-synthesized high power function generator has
several advantages. First, very complicated wave forms
can be generated to optimize the fusion and/or poration of
different types of cells. Second, when the high power
function generator is used in more than one protocol or by
35 more than one user, each protocol can be stored separately

1 in a data storage device for example, a magnetic
diskette. Since the protocols can be recalled quickly,
the high power function generator can be reprogrammed to
5 generate the desired wave forms without manually
readjusting all the parameters. Third, the same computer
can be used as a digital oscilloscope to record the actual
electrical field applied to the cells. This record can be
saved in a data storage device as the permanent record of
10 any particular cell fusion or cell poration experiment.

Excessive current is harmful to the cell because
of the resulting thermal effects and pH changes. To avoid
generating excessive current and the resulting effects
during the application of the electric field, the
15 suspension medium of the cells is usually a low ionic
strength solution. Preferably it contains very low
concentration of salts. A typical suspension medium may
contain 1 mM of electrolyte including 0.4 mM Mg-acetate
and 0.1 mM Ca-acetate. The medium is buffered and the pH
20 maintained in the physiological range, for example, pH
7.5. Any buffer commonly used for biological purposes,
for example, 1 mM HEPES

(N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) is
adequate for cell poration and/or cell fusion.
25 Non-electrolytes are added to maintain the osmolarity of
the medium at about the osmolarity of extracellular
fluid. In the preferred embodiment, relatively high
molecular weight, cell impermeable carbohydrates, such as
sucrose and mannitol, are used to maintain the osmolarity.

30 For some cells, a slightly higher ionic strength
in the medium seems to improve the fusion yield. For
example, human erythrocytes fuse easily in 30mM
Na-phosphate. Thus, the present method of fusion can use
suspension medium with an ionic strength ranging from 0.1
35 mM to 100mM depending on the cell type.

The present invention for cell poration and cell

1 fusion has a variety of uses. Many biological active
substances, including DNA, RNA, organic chemicals,
inorganic chemicals, drugs, antibodies, proteins,
5 hormones, growth factors, enzymes and radio- or
fluorescent-labelled molecular probes normally cannot be
readily taken up by cells. The present invention provides
an effective method to transport these biological active
substances into the cells. In one embodiment of the
10 present invention, cells can be temporarily permeabilized,
that is porated, by applying high-power RF pulses and the
biological active substances can then enter the cells
during this poration period. The porated cells can be
biological cells (including, animal, human or plant
15 cells), protoplasts, bacteria or yeasts. In another
embodiment of the present invention, the biological active
substances can be inserted into the cells by fusing the
target cells with other biological particles which have
been pre-loaded with the active substances. Such
20 biological particles include liposomes and erythrocyte
ghosts, which can be easily preloaded with desired
substances using a standard osmotic shock and dialysis
method. (Schlegel & Lieber Cell Fusion ed by A.E. Sowers
Plenum Press (1987)). The target cells may be any cells
25 which will receive the biological active substances and
include isolated cells, egg cells, embryonic cells, any
primary or transformed cultured cells, or other cells in
vitro.

In like manner, biological substances could be
30 extracted from biological cells. For example, many
molecules such as hormones, growth factors, enzymes,
proteins and nucleic acids may not be able to cross the
membrane barrier. Using the poration method of the
present invention, temporary pores can be induced in the
cell membrane. The non-permeable molecules can then exit
35 the cell. This procedure could be useful in a variety of

1 industries which use growing cells to produce biological
molecules. This procedure allows the extraction of these
molecules without having to kill the cells.

Example I

5 The ability and efficiency of the RF
electroporation method to insert foreign genes into the
target cell is examined using the cultured eukaryotic
fibroblast cell line COS-M6 (M6). Chloramphenicol
10 AcetylTransferase (CAT) DNA was used as a gene marker.
Bacterial CAT DNA was inserted into a plasmid vector
(pSV₂-CAT). The CAT enzyme is not endogenously produced
in mammalian cells, such as M6. Thus, the amount of CAT
gene incorporated into the target cells can be assayed by
15 monitoring the amount of CAT enzyme produced after the
transfection.

The protocol was to apply 3 trains of high-power
RF pulses at 10 sec intervals. Each train consists of 5
pulses (frequency 100 KHz, field strength 2.5 KV/cm, pulse
20 width 0.5 msec).

The RF poration protocol of the present invention
is a highly effective method of gene transfection. In the
conventional methods of gene transfection, for example,
the calcium phosphate method or the DEAE-dextran method,
25 usually requires at least 5-10 µg of plasmid DNA for
each transfection. In previous electroporation methods
that used DC pulses, even larger amounts of DNA (typically
10-40 µg) were required. (Ansubel et al., Current
Protocols in Molecular Biology, John Wiley & Sons, 1988).
30 Using the RF poration method of this invention, we
obtained a high level of CAT activity (76% acetylation per
25 µg of protein) when M6 cells were transfected using
only 0.1 µg of CAT DNA. Furthermore, up to 10.6%
acetylation per 25 µg of protein was observed when M6
35 cells were transfected with as little as 0.01µg
of CAT DNA. Thus, it is evident that the RF poration

1 method has a much higher efficiency of gene transfection.
The improved efficiency not only results in great savings
in labor and material that is required to produce DNA, but
5 also will allow the transfection of cells which were
previously difficult to transfect.

Another advantage of the RF poration method is
that it requires far less cells for gene transfection.
The conventional chemical methods and the DC
10 electroporation method typically require 2 to 10 million
cells to do one transfection. With the RF method, M6
cells have been transfected with the CAT gene in high
efficiency using as few as 0.1 million cells. Further
experiments indicated that even lower numbers of cells (1
15 $\times 10^4$) can be used. Currently, the minimum cell number
is limited by the amount of total cell protein required to
perform the CAT assay and not the ability to transfect
cells. (Typically 25 micrograms of total cellular protein
are needed for the CAT reaction.)

20 Example II

Because of the unique abilities of the RF
poration method to transfect cells in small quantity and
with high efficiency, the method will be particularly
useful in the development of gene therapy. Many diseases
25 are known to be caused by genetic defects. Such diseases
could be treated by inserting a therapeutic gene into
human cells such as bone marrow stem cells and then
transplanting these cells into the human body.

For example, patients with sickle cell anemia
30 have a defective gene which produces abnormal hemoglobin.
To treat such a genetic disease, bone marrow stem cells
are extracted from the patient and transfected with the
normal hemoglobin gene. The transfected stem cells are
transplanted back into the patient. With the appropriate
35 vector the normal gene will be stably integrated into the
genome and the patient will be able to produce normal

1 hemoglobin.

The key step in this treatment is the transfection of the bone marrow stem cells with the normal gene. Because the number of stem cells which are extracted is relatively small, a gene transfection method of high efficiency that is suitable for extremely low cell numbers is required. The method of the present invention of poration using RF pulses uniquely has this ability. Thus this method will be highly useful for gene therapy.

10 The usefulness of this method for gene therapy is not limited to sickle cell anemia. This method can be applied to insert normal genes into human cells to cure many genetic diseases. Other examples include: introducing the gene for clotting factor VIII into bone marrow stem cells to cure hemophiliacs; inserting the gene for insulin into pancreatic islet cells or other human cells to treat diabetes; introducing the gene for the human LDL (low density lipoprotein) receptor into liver cells or other human cells to lower the cholesterol level in the bloodstream of hypercholesterolemia patients; and introducing the gene for human growth hormone into human cells to correct growth defects. Thus, the possibilities of using this method to insert genes into human cells to treat genetic diseases is unlimited.

Example III

Morphological Changes of the Cell Membrane during the Process of RF Field Electroporation

30 A fraction of a second after human red blood cells were exposed to RF pulses, they were rapidly frozen in liquid freon cooled by liquid nitrogen. The structure of the cell membranes were examined using the technique of freeze-fracture electron microscopy. In Fig. 14 the electron micrograph shows the surface structure of the red blood cell after 3 RF pulses (400 kHz, 40 μ sec wide, 5

1 kV/cm field strength) were applied. Membrane pores with
diameters of 0.1 to 0.3 micrometers were clearly seen.
These pores are sufficiently large to allow a large piece
5 of DNA to easily diffuse from the extracellular medium
into the cell. Thus, there is direct evidence that the
applied RF fields can induce large pores at the cell
surface. The morphological evidence clearly shows that
the method of the present invention is effective in
10 inducing membrane poration to allow transfection of cells
with exogeneous genes.

Example IV

An example of the advantage that the present
invention has over the conventional DC (direct current)
15 electrofusion method was seen in the fusion of human
erythrocytes. The fusion events were assayed by labelling
the membranes of a small number of the suspended cells
with a lipophyllic fluorescent dye, for example,
1,1',-dihexadecyl-3,3,3",3'-tetramethylendocarbocyanine
20 perchlorate. The cells were observed with a fluorescence
microscope. Before applying the RF pulses, only the
prelabelled cells give a fluorescent image and they
appeared as isolated cells (see Figure 16A). After the
cells were exposed to pulsed RF fields, unlabelled cells
25 started to fuse with labelled cells and the dye was
gradually transferred from the labelled cell to the
unlabelled cell. Eventually both cells became labelled
(see Figure 16B). This fusion process took only a few
minutes following the application of the RF pulses.

Two types of cell fusion were observed in this
30 experiment: (1) Membrane fusion, in which the fluorescent
dye was transferred from the labelled cell to the
unlabelled cell but the two cells did not merge their
cytoplasm; and (2) cytoplasmic fusion, in which the fusing
cells merged together to form one single large cell. The
35 percentage of cells undergoing cytoplasmic fusion depends

1 strongly on the oscillating frequency of the applied RF
field. The fusion yield for erythrocytes after RF pulses
of different frequency are applied is shown in Figure 17.
5 The highest yield of fused cells occurred when the applied
RF field was oscillating at 100KHz. The fusion yield
decreased to a very low level as the frequency became too
high or too low. No cytoplasmic fusion was detected when
the applied field was in the form of DC pulses with the
10 same pulse amplitude and pulse width as the RF pulses.
These results clearly indicate that the RF pulse method of
this invention is much more effective in inducing cell
fusion than the DC pulse method.

Another example of the advantage of the present
15 invention over the DC electrofusion method is in the
fusion of human erythrocytes with a human leukemia
cultured cell line, HL-60. Fusion of these two cells
types was not obtainable using the DC pulse method. The
failure is probably due to the differences in cell size;
20 erythrocytes are significantly smaller than HL-60 cells.
However, using the fluorescent dye assay and the pulsed RF
field of the present invention, we were able to obtain the
fusion of erythrocytes with HL-60 cells.

Example V

25 The RF pulse method can be used to fuse cells to
make hybridomas. Pigment cells from goldfish
(xanthophores) were fused with a tumor cell line derived
from fish skin cells. Because xanthophore cells have a
built-in histochemical marker (the carotenoid droplets),
30 it is comparatively easy to assay their fusion with
non-pigmented tumor cells. Fig. 18 shows the sequential
steps in the fusion of a xanthophore and a skin tumor
cell. In Fig. 18A the cells were brought into close
contact by dielectrophoresis. Three pulses of RF field
35 (40 μ sec wide, frequency 400 kHz, field strength 3.3
kV/cm) were then applied. Within two minutes the

1 cytoplasms of the two cells begun to merge (see Figure 18B). After 4 minutes, the cells completely coalesced into a single giant cell (see Figure 18C).

5 An important application of forming hybridomas using the RF pulse method is to make antibodies, especially human monoclonal antibodies. In this instance the biological particles to be fused can include antibody producing cells (for example, lymphocyte B cells) and
10 continuously dividing cells (for example, cancer cells). Using a selection process, the resultant hybridoma cells can be cultured to produce specific monoclonal antibodies.

One skilled in the art will readily appreciate
15 the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The devices, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended
20 to be exemplary, and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

25 What is claimed is:

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35

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CLAIMS

1. A method for poration or permeabilization of
5 biological particles, comprising the steps of:
placing a plurality of biological particles
between electrodes; and
applying a pulsed radiofrequency oscillating
electrical field across said electrodes.

10

2. The method of Claim 1, wherein said
biological particles are selected from the group
consisting of animal cells, human cells, plant cells,
protoplasts, bacteria and yeasts.

15

3. The method of Claim 1, for injecting
chemical agents or biologically active molecules into
biological cells, wherein said chemical agents or
biologically active molecules are selected from the group
20 consisting of DNA, RNA, antibodies, proteins, drugs,
hormones, growth factors, enzymes, organic chemicals, and
inorganic chemicals.

4. The method of Claim 1, for extracting
25 molecules from biological cells, wherein said molecules
are selected from the group consisting of proteins,
nucleic acids, hormones, growth factors, enzymes, and
other biologically active molecules.

5. The method of Claim 1, wherein said pulsed
30 radiofrequency field includes a frequency range of about
10 KHz to 100 MHz, a pulse width range of about 1 μ sec
to 10 msec, and a pulse amplitude range of about 1 KV/cm
to 20 KV/cm.

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6. . The method of claim 5 wherein the pulses are selected from the group consisting of single pulses, train of pulses and multiple train of pulses.

5

7. The method of Claim 1, wherein said pulsed radiofrequency oscillating field includes a frequency of about 0.02 to 10 MHz; a pulse width of about 20 to 2000 μ sec and a pulse amplitude of 2-10 KV/cm.

10

8. The method of Claim 1, wherein said pulsed radiofrequency oscillating field includes different wave forms.

15

9. A method for fusing biological particles, comprising the steps of:

placing a plurality of biological particles in solution between electrodes;

positioning said particles in close proximity; and

20

fusing said biological particles by applying a pulsed radiofrequency oscillating electrical field across said electrodes.

25

10. The method of Claim 9, wherein said positioning step includes placing suspended biological particles in a container which allows said biological particles to congregate.

30

11. The method of Claim 9, wherein said positioning step includes:

applying an alternating electric field across said electrodes for bringing said particles into close proximity.

35

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12. The method of Claim 11, wherein said
applying an alternating electrical field occurs before and
after said fusing step.

5

13. The method of Claim 9, wherein said
biological particles include a target cell and a particle
preloaded with chemical agents or molecules for
10 introducing chemical agents and molecules into cells.

15

14. The method of Claim 13, wherein:
said particle preloaded with said chemical agents
or molecules is selected from the group consisting of
erythrocyte ghosts, liposomes, vesicles, isolated
cells and cultured cells; and
said target cell is selected from the group
consisting of animal cells, human cells, plant cells,
bacteria and yeast.

20

15. The method of Claim 13, wherein said
chemical agents and molecules are selected from the group
consisting of antibodies, proteins, drugs, molecular
probes, hormones, growth factors, DNA, RNA, enzymes,
25 organic chemicals and inorganic chemicals.

30

16. The method of Claim 9, wherein said
biological particles include cells from at least two
different species.

17. The method of Claim 9, wherein said
biological particles include an antibody producing cell
and a continuously dividing cancer cell.

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18. The method of Claim 9, wherein said biological particles include hyperimmunized mouse spleen cells and myeloma cells.

5

19. The method of claim 9, wherein said biological particles include human lymphocytes and human cancer cells.

10

20. The method of Claim 9, wherein said biological particles include cells of at least two different types.

15

21. A method for fusing biological particles, comprising the steps of:

placing a plurality of biological particles in solution between electrodes, wherein said biological particles are selected from the group consisting of animal cells, human cells, plant cells, vesicles, erythrocyte ghosts, liposomes, protoplasts, bacteria and yeasts;

providing a low amplitude alternating electrical field across said electrodes for bringing said particles into close proximity by dielectrophoresis, wherein said electrical field has a frequency range of about 60 Hz to about 10 mega Hz and a field strength of about 100 to 800 V/cm.;

applying a pulsed single or multi-frequency radiofrequency oscillating electrical field across said electrodes for fusing said particles, wherein said radiofrequency oscillating electrical field includes a frequency range of about 10 KHz to 100 MHz; a pulse width range of about 1 μ sec to 10 msec, and a pulse amplitude range of about 1 KV/cm to 20 KV/cm; and

35

1 providing an alternating electrical field across
said electrodes for bringing said particles into close
proximity for fusion, wherein said electrical field
5 has a frequency range of about 60 Hz to about 10 mega
Hz and a field strength of about 100 V/cm to 800 V/cm.

22. As a composition of matter, a hybridoma cell
made by a method for fusing antibody producing cells with
10 continuously dividing cancer cells, comprising the steps
of:

suspending antibody producing cells and cancer
cells in solution between electrodes;
providing an alternating electrical field wherein
15 said electric field forms pearl chains by allowing the
cells to move dielectrophoretically; and
forming said hybridoma cell by applying a high
strength pulsed radiofrequency oscillating field
across said electrodes.

20 23. The hybridoma cell of Claim 22, wherein said
antibody producing cells are hyperimmunized mouse spleen
cells and the cancer cells are myeloma cells.

25 24. The hybridoma cell of claim 22, wherein said
antibody producing cells are human lymphocytes and the
cancer cells are human cell lines.

25. A method of inserting foreign genes into
30 biological particles comprising the steps of:
placing a plurality of biological particles
between electrodes in a solution containing the
foreign gene; and
applying a pulsed radiofrequency oscillating
35 electrical field across said electrodes.

1

26. The method of claim 25, wherein,
said pulsed radiofrequency includes a frequency
range of about 10 KHz to 10 MHz, a pulse width range
of about 1 μ sec to 10 msec and a pulse amplitude of
about 1 to 20 KV/cm;

5

said biological particles are selected from the
group consisting of human cells, animal cells, plant
cells, protoplasts, bacteria and yeasts.

10

27. A method of treating genetic diseases,
comprising the steps of:

15

inserting in vitro a gene into cells by the
method of claim 25; and

transplanting said cells with the inserted gene
into the organism with the genetic defect.

20

28. The method of claim 27, wherein the organism
is a human.

25

29. The method of claim 27, wherein, the cells
in which the gene is inserted are extracted from the
organism with the genetic defect prior to the insertion
steps.

30

30. The method of claim 27, wherein, the genetic
disease is sickle cell anemia, the cells are bone marrow
stem cells and the gene is the normal hemoglobin gene.

35

31. The method of claim 27, wherein,
the genetic disease is selected from the group
consisting of hemophilia, diabetes,
hypercholesterolemia and growth disorders;

1 the cells are selected from the group consisting
of bone marrow stem cells, pancreatic islet cells,
liver cells and other human cells; and

5 the gene is selected from the group consisting of
the normal clotting factor VIII gene, the normal
insulin gene the normal low density lipoprotein
receptor gene and the growth hormone gene.

10 32. The method of Claim 27, wherein said cells
are bone marrow stem cells.

 33. A device for the poration and fusion of
biological particles comprising:

15 a container, including a non-conducting material
capable of holding liquid, wherein said container
includes an access port for receiving said biological
particles;

 electrodes positioned equidistant from each other
in said container; and

20 a high power function generator attached to said
electrodes for applying an electrical field including
a pulsed radiofrequency electric field.

25 34. The device of Claim 33, wherein said
container has a shape which allows the biological
particles to congregate by gravity.

30 35. The device of Claim 33, wherein said
radiofrequency is a pulsed radiofrequency oscillating
field and includes a frequency range of about 10 KHz to
100 MHz; a pulse width range of about 1 μ sec to 10 msec;
and a pulse amplitude range of about 1 KV/cm to 20 KV/cm.

35 36. The device of Claim 33, wherein said
function generator further includes the capability of

1 applying a continuous low power alternating current for
bringing said biological particles into close proximity
for fusion.

5 37. The device of Claim 36, wherein said low
power alternating current includes a frequency range of
about 60 Hz to the 10 mega Hz range and a field strength
of about 100 V/cm to 800 V/cm.

10 38. A device for optical microscopic observation
of poration and fusion of biological particles comprising:
a container with transparent bottom;
electrodes inserted into said container, wherein
15 said electrodes are equidistant from each other and
arranged in a pattern which allows at least ten
microliter of cells to closely contact the electrodes;
and
a high power function generator attached to said
20 electrodes for applying an electrical field including
radiofrequency.

39. A device for cell poration and fusion of
biological particles, comprising:
25 a handle of non-conductive material for
manipulating said device;
electrodes positioned equidistant from each other
and attached to said handle; and
a connecting means for attaching the electrodes
30 to a high power function generator.

40. The device of Claim 39, wherein said
electrodes are wrapped around a non-conducting core in the
form of a double helix.

35

1 41. The device of Claim 39, wherein said
electrodes comprise:
 segmented shapes; and
5 insulating spacers for separating said segmented
 shapes.

 42. The device of Claim 39, wherein said
electrodes are bottom-contact electrodes selected from the
10 group consisting of spirals, concentric rings, concentric
squares, parallel plates and interdigitating arrays.

 43. The device of claim 39, wherein said
electrodes are coaxial and are about 0.5 to 2.0 mm apart,
15 have a length of about 0.5 to 3 cm and a diameter
sufficiently small to fit into a well of a multi-well
culture plate.

 44. A high power function generator, comprising:
20 a RF pulse generator including a gating circuit
for gating the output of a radiofrequency oscillator
and a power amplifier for generating the high power RF
pulse from the gated output of the radiofrequency
oscillator;
25 an AC field generator; and
 a mercury wetted relay for switching between the
RF pulse and the AC field.

 45. A device for cell poration and cell fusion,
30 comprising:
 a digital computer for synthesizing and
generating a RF wave form and an AC wave form;
 a amplifier to convert the wave form generated by
the digital computer to high power wave forms; and
35 an electrode communicating with said

1 amplifier for applying the electric field to
 biological cells.

5 46. The device of claim 45 further including an
 information storage device.

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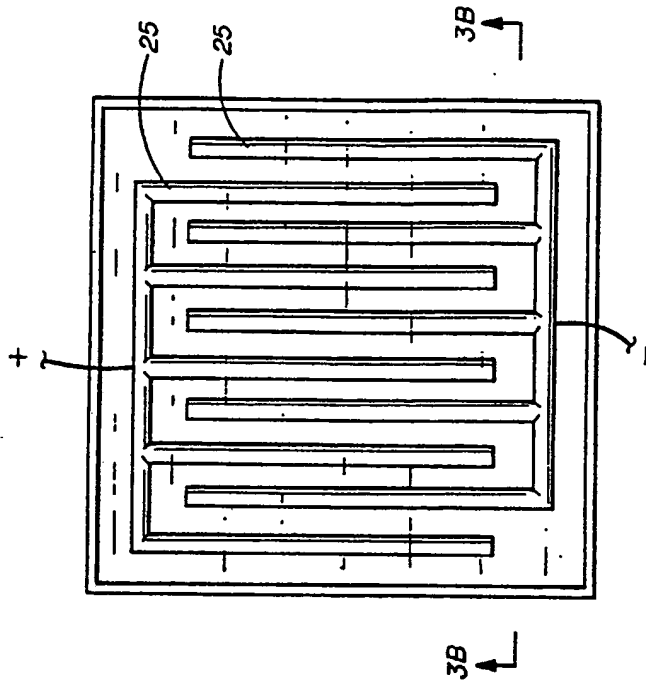


FIG. 3A

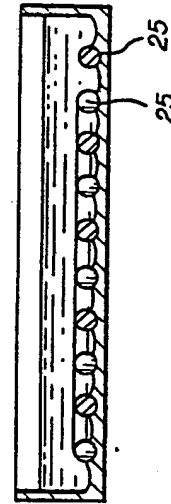


FIG. 3B

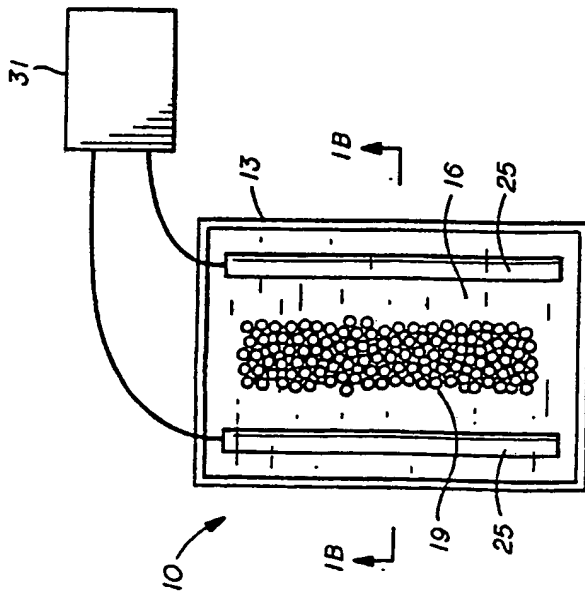


FIG. 1A

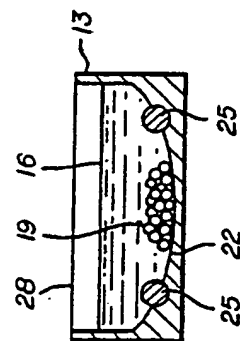
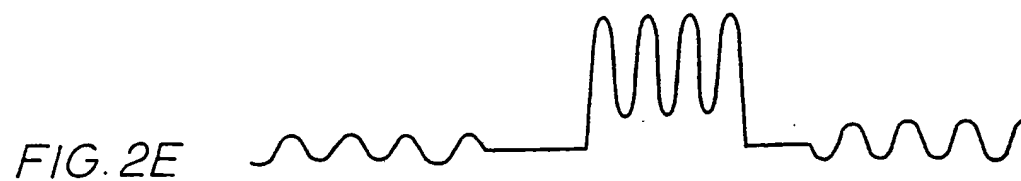
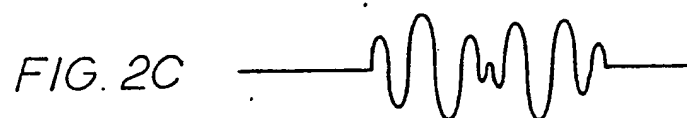


FIG. 1B

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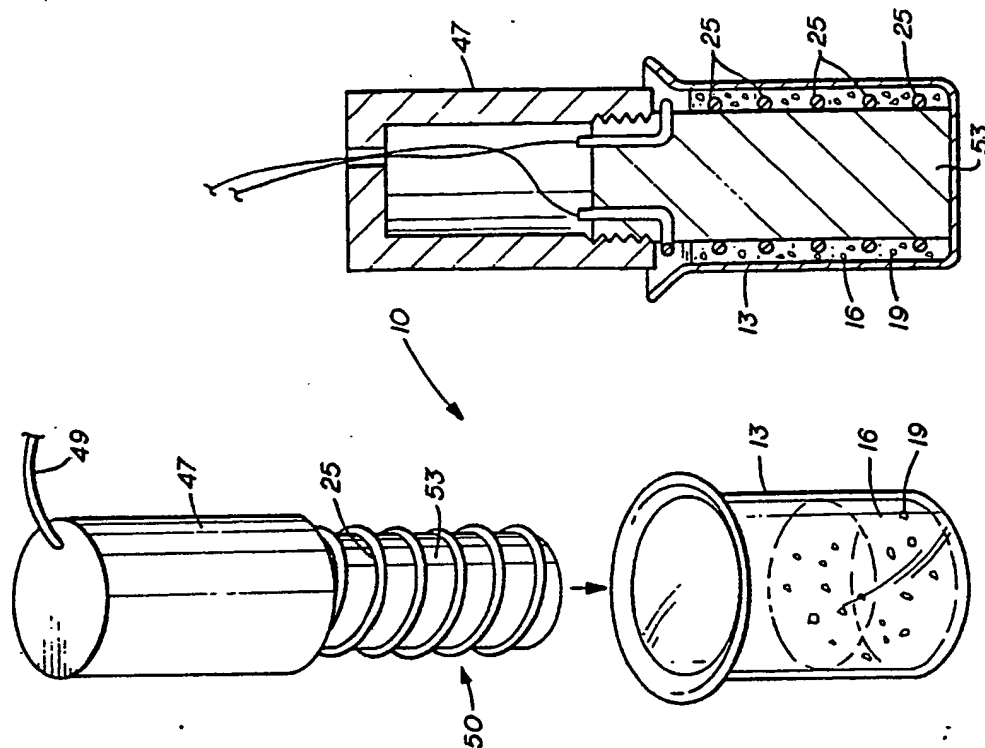


FIG. 5B

FIG. 5A

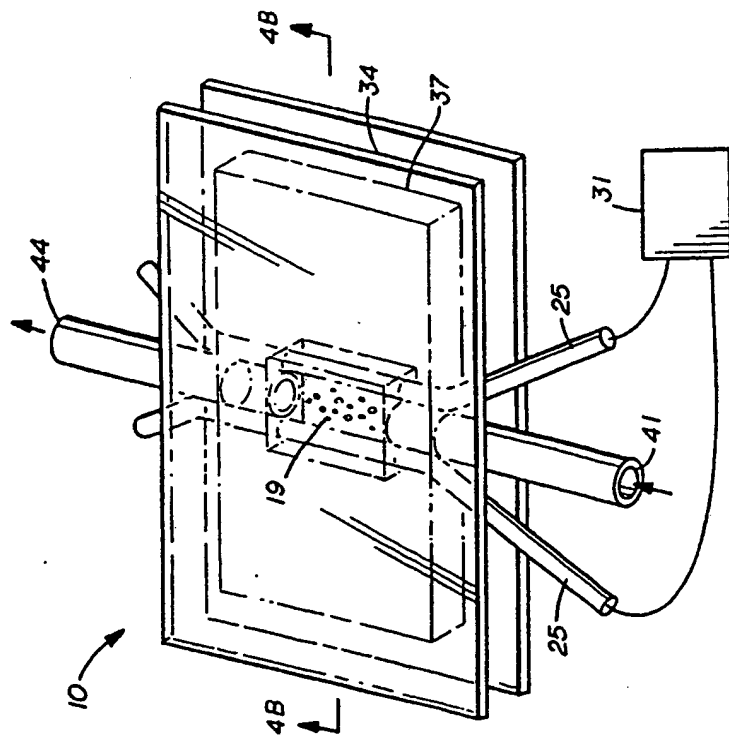


FIG. 4A

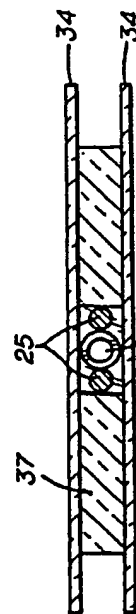


FIG. 4B

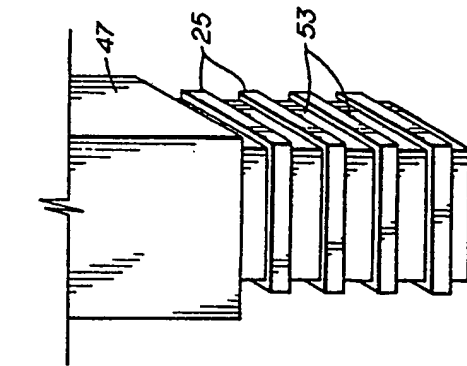


FIG. 8A

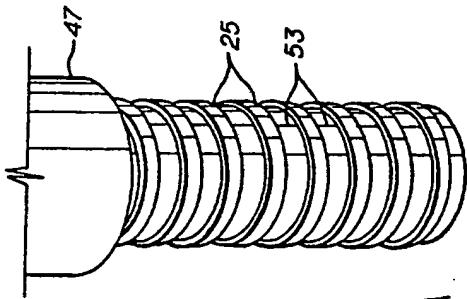


FIG. 7A

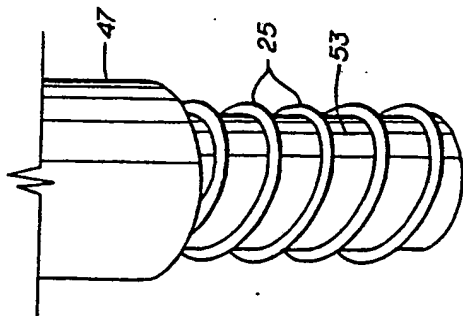


FIG. 6A

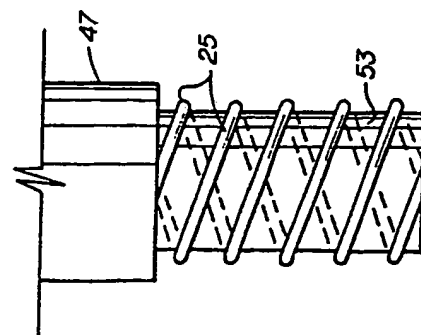


FIG. 6B

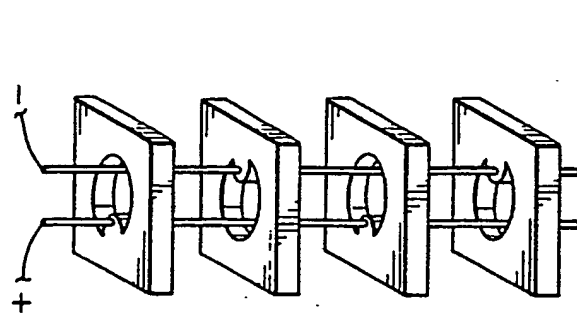


FIG. 8B

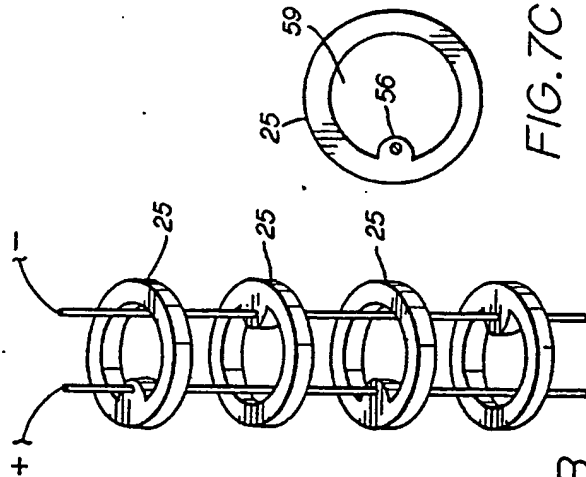


FIG. 7B

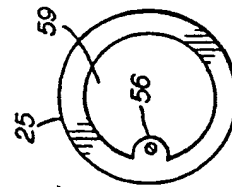


FIG. 7C

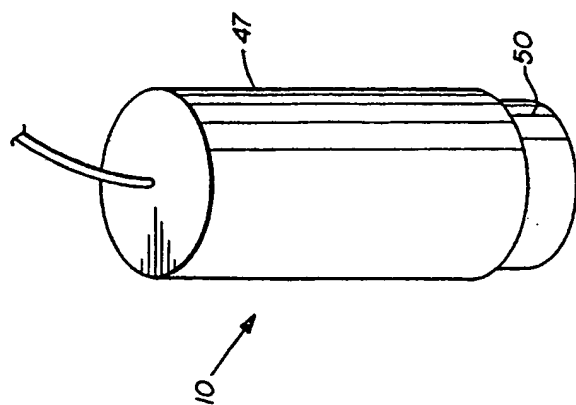


FIG. 9

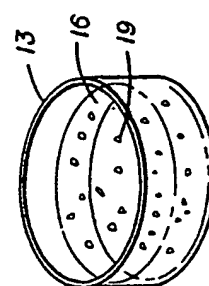


FIG. 10A

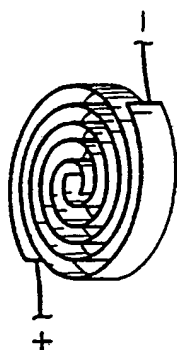


FIG. 10B



FIG. 11A

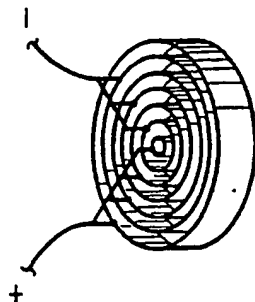
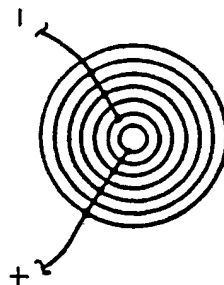


FIG. 11B



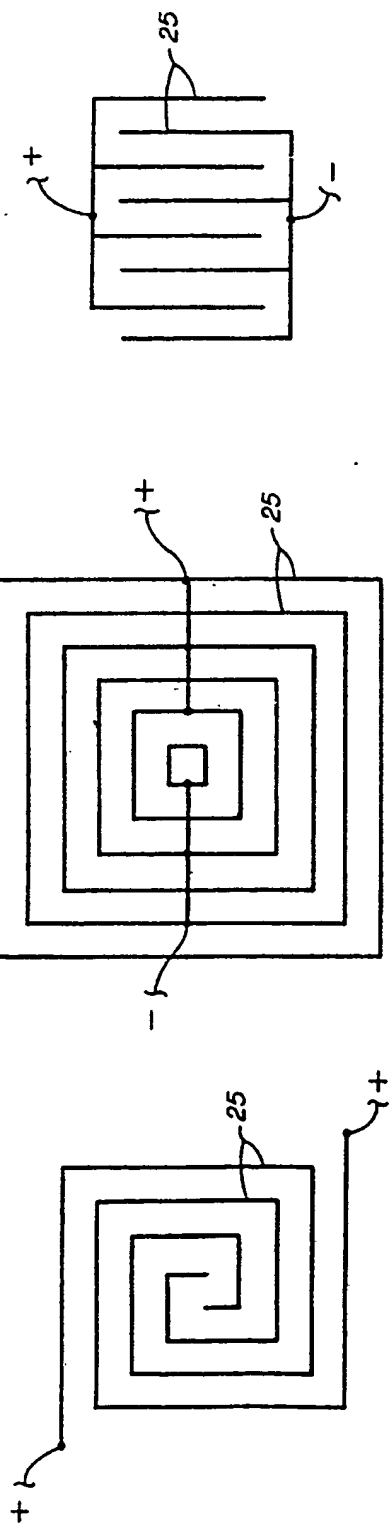


FIG. 12A

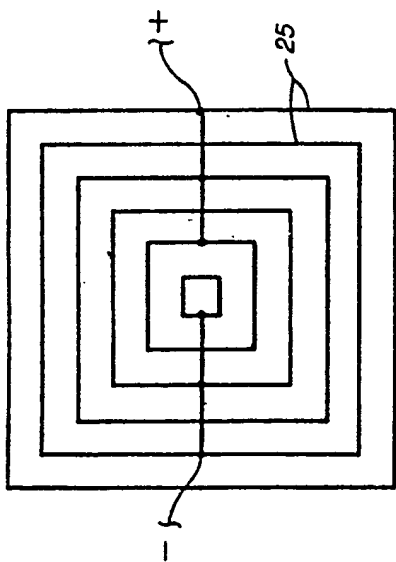


FIG. 12B

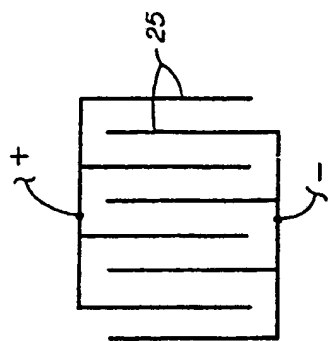


FIG. 12C

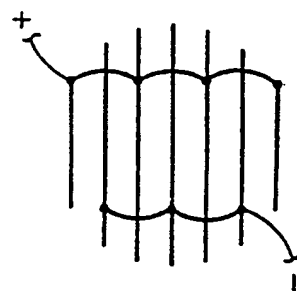


FIG. 12D

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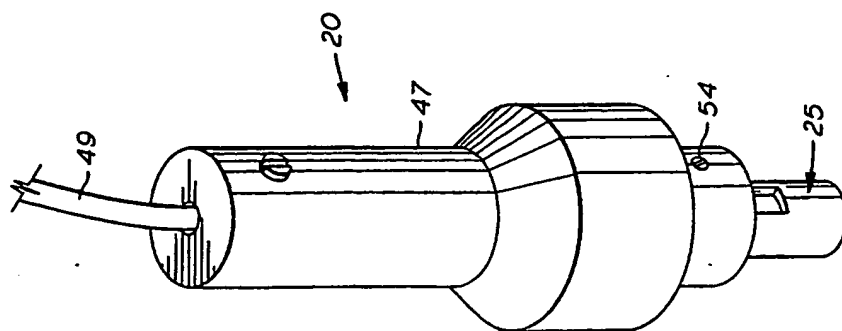


FIG. 13A

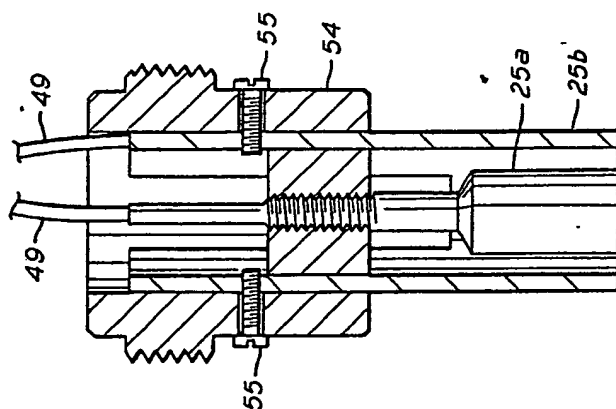


FIG. 13B

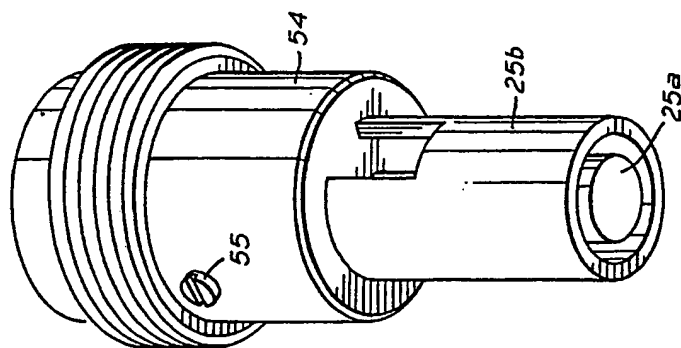


FIG. 13C

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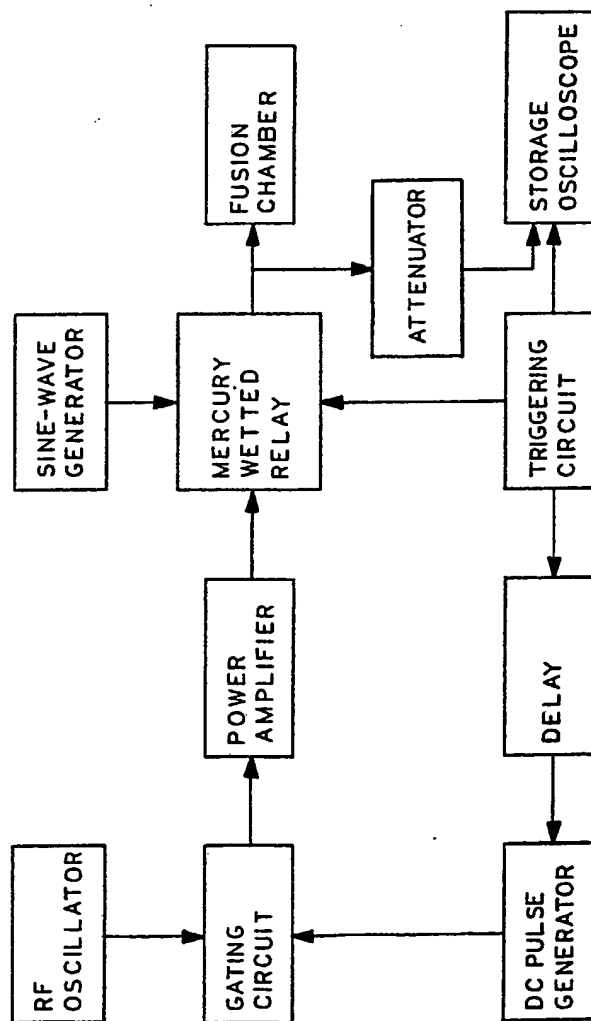


FIG. 14

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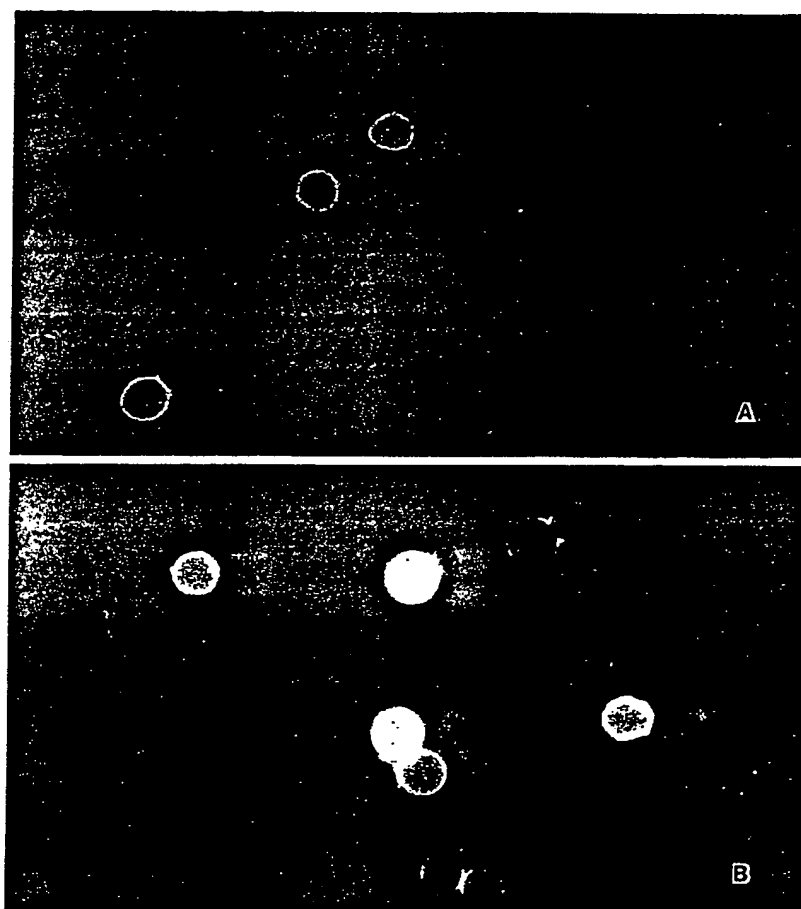


FIG. 15

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FIG. 16

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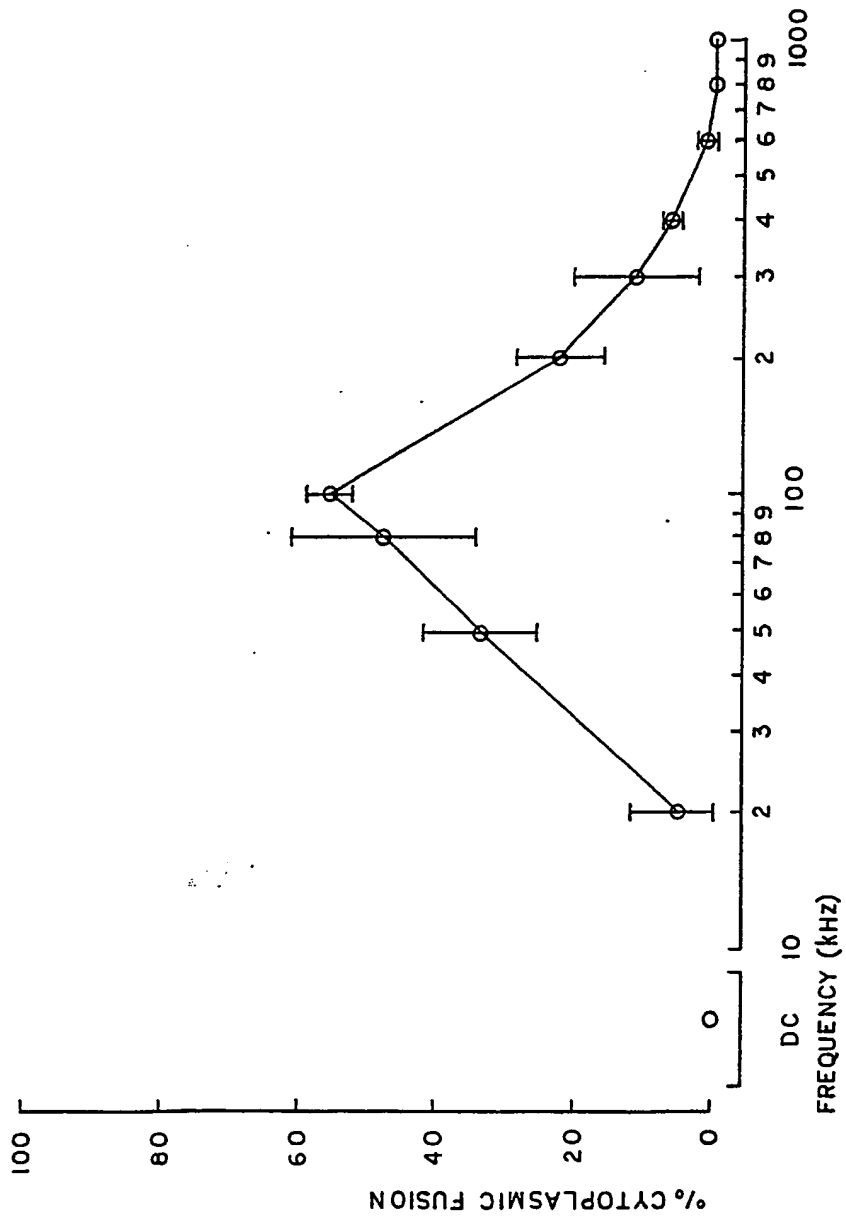


FIG. 17

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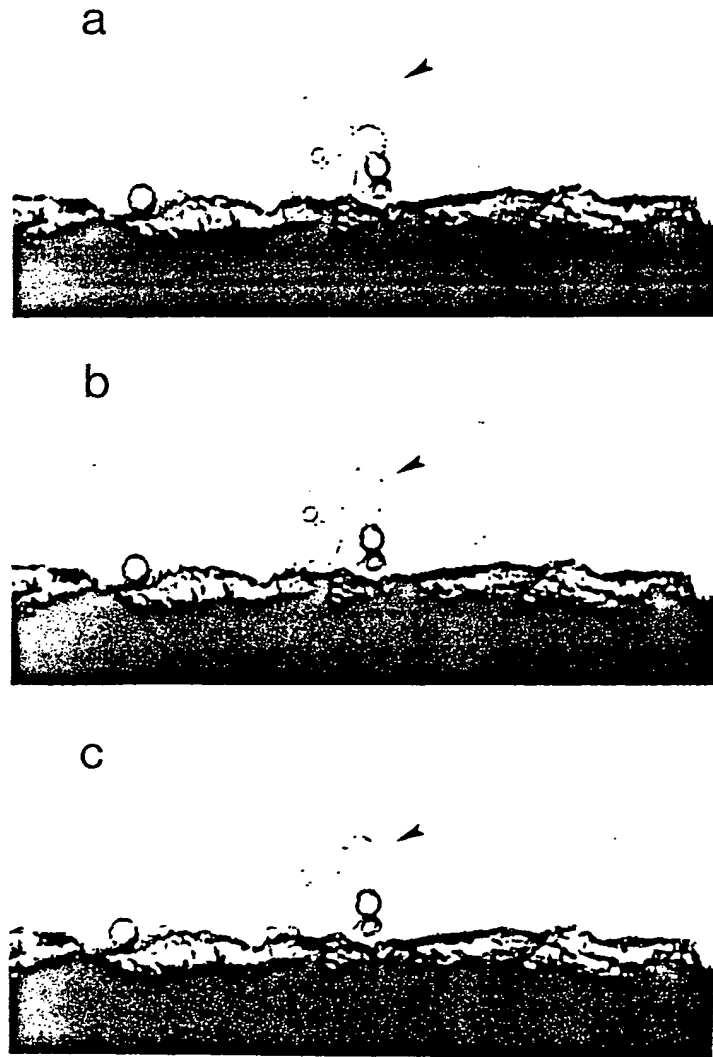


FIG. 18



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12N 15/00, 13/00	A3	(11) International Publication Number: WO 89/ 03426 (43) International Publication Date: 20 April 1989 (20.04.89)
(21) International Application Number: PCT/US88/03457 (22) International Filing Date: 5 October 1988 (05.10.88) (31) Priority Application Numbers: 106,282 238,607 (32) Priority Dates: 9 October 1987 (09.10.87) 30 August 1988 (30.08.88) (33) Priority Country: US (71) Applicant: BAYLOR COLLEGE OF MEDICINE [US/ US]; One Baylor Plaza, Houston, TX 77030 (US). (72) Inventor: CHANG, Donald, C. ; 6306 Coachwood, Houston, TX 77035 (US). (74) Agent: PAUL, Thomas, D.; Fulbright & Jaworski, 1301 McKinney St., #5100, Houston, TX 77010 (US).	(81) Designated States: AT (European patent), AU, BE (Eu- ropean patent), CH (European patent), DE (Euro- pean patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent). Published <i>With international search report</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> (88) Date of publication of the international search report: 13 July 1989 (13.07.89)	
(54) Title: METHOD OF AND APPARATUS FOR CELL PORATION AND CELL FUSION USING RADIOFRE- QUENCY ELECTRICAL PULSES		
<div data-bbox="250 1163 380 1194" data-label="Section-Header">(57) Abstract</div> <div data-bbox="250 1215 649 1856" data-label="Text"> <p>Disclosed are an apparatus and a method for the poration and fusion of cells using high-power radiofrequency electrical pulses. The electrodes of the apparatus can be hand held or part of integrated equipment with special containers for cells. The electrodes, which are positioned equidistant from each other, are attached to a high power function generator. The power function generator can apply a continuous AC electrical field and/or a high-power pulsed radiofrequency electrical field across the electrodes. The alternating electrical field induces cell congregation by the process of dielectrophoresis. The high-power pulsed radiofrequency electrical field porates or fuses the cells. The method has the ability to porate or fuse biological cells with a very high efficiency. The method can be used to fuse or porate a variety of cells including animal cells, human cells, plant cells, protoplasts, erythrocyte ghosts, liposomes, vesicles, bacteria and yeasts. The method has the unique ability to porate or fuse cells in very small or very large numbers. During the poration or fusions, a variety of chemical agents including DNA, RNA, antibodies, proteins, drugs, molecular probes, hormones, growth factors, enzymes, organic chemicals and inorganic chemicals can be introduced into these cells. The method can also be used to produce new biological species, to make hybridoma cells which produce animal or human monoclonal antibodies and to insert therapeutic genes into human cells which can be transplanted back into the human body to cure genetic diseases.</p> </div> <div data-bbox="688 1121 1159 1604" data-label="Image"> </div>		

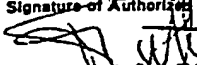
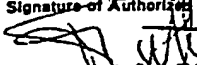
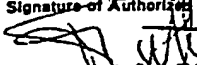
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FI	Finland				

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03457

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00; C 12 N 13/00														
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <div style="display: flex; justify-content: space-between;"> <div style="width: 30%; border-bottom: 1px solid black; padding-bottom: 5px;">Classification System ¹</div> <div style="width: 65%; border-bottom: 1px solid black; padding-bottom: 5px;">Classification Symbols</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%; border-right: 1px solid black; padding-right: 5px;">IPC⁴</div> <div style="width: 65%; padding-left: 5px;">C 12 N</div> </div> <div style="border-top: 1px solid black; padding-top: 5px; margin-top: 10px;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ </div>														
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Category ⁹	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
A	EP, A, 0128567 (KERNFORSCHUNGSANLAGE JULICH GmbH) 19 December 1984, see claims --	1-4, 11												
A	US, A, 4578168 (G.A. NOFMANN) 25 March 1986, see claims; column 5 --	1												
A	US; A, 3059359 (J.H. HELLER) 25 June 1963, see claims -----	1, 5												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">30th May 1989</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">19 JUN 1989</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">  P.C.G. VAN DER PIJPEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">30th May 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">19 JUN 1989</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">  P.C.G. VAN DER PIJPEN </div>								
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International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">  P.C.G. VAN DER PIJPEN </div>													

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
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US 8803457
SA 25321

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The members are as contained in the European Patent Office EDP file on 13/06/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0128567	19-12-84	DE-A,C 3321226 JP-A- 60009490	13-12-84 18-01-85
US-A- 4578168	25-03-86	JP-A- 61037090	21-02-86
US-A- 3059359		None	

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